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TECHNICAL MANUSCRIPT 406

ABSCISSION: INDUCTION OF DEGRADATIVE ENZYMES DURING AGING

Frederick B. Abeles
Robert E. Holm
Harry E. Gahagan, III

JULY 1967

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BIOLOGICAL SCIENCES LABORATORY

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ABSTRACT

Loss of weight, chlorophyll, RNA, and protein from pulvinal tissue of Phaseolus vulgaris L. var. Red Kidney petiole explants was inhibited by actinomycin D or cycloheximide. The loss of RNA was correlated with the induction of ribonuclease. Concentrations of auxin, cytokinins, coumarin, actinomycin D, or cycloheximide sufficient to maintain levels of RNA in pulvinal tissue inhibited the induction of ribonuclease. Evidence is presented that lysosomes are not the source of ribonuclease. Abscission retardants are probably aging retardants, and the loss of metabolites from the pulvinus and induction of cell separation by ethylene are a part of the aging phenomenon.

I. INTRODUCTION

Scott and Leopold[1] and Abeles et al.[2] have shown that there is a loss of chlorophyll, RNA, and protein from the pulvinus of bean petiole explants after excision and that this loss can be inhibited by auxin, cytokinins, or coumarin. It is also known that auxin,[1] cytokinins,[3] and coumarin[2,4,5] retard aging when the process is measured as a loss of RNA and other metabolites from plant tissue. The work reported here suggests that: (i) catabolic enzymes are synthesized in the pulvinus after an aging period and (ii) auxin, cytokinin, and coumarin function as aging retardants, thereby repressing the synthesis of catabolic enzymes. Because aging is also a prerequisite for ethylene action[6-8] in abscission, the retardation of aging by auxin, cytokinins, and coumarin probably accounts for their ability to prevent abscission.

II. MATERIALS AND METHODS

Seeds of Phaseolus vulgaris L. var. Red Kidney sown in 10-cm pots filled with soil were grown for 14 days at 26±2 C at 1,200 ft-c of fluorescent light (12-hour photoperiod). Abscission zone explants from the primary leaves were incubated in bottles (43±2 ml in volume, 5 cm in diameter, and 2.5 cm high, fitted with 25-mm diameter rubber vaccine caps) at 25 C in 400 ft-c of continuous fluorescent light. The 10-mm-long explants (4.5 mm of pulvinal tissue) were placed, pulvinal end down, in 3 mm of 1.5% agar. Explants were treated by incorporating various growth regulators into the agar. One µliter of a solution of actinomycin D or cycloheximide was injected into the separation zone of explants with a microliter syringe. Injection of 1 µliter of water had no effect on abscission compared with untreated controls. When required, ethylene was added to the gas phase with a syringe inserted through the vaccine cap.

A. DRY WEIGHT

Changes in the dry weight of pulvinal tissue were determined after the tissue had dried at 98 C for 16 hours.

B. CHLOROPHYLL

Ten petiole sections were homogenized in 10 ml of methanol for 2 minutes at high speed in a VirTis homogenizer fitted with a 50-ml flask.

The homogenate was filtered through Miracloth* and centrifuged at 2,000 x g for 10 minutes. Chlorophyll was determined by measuring the optical density of the solution at 666 mμ in a spectrophotometer.

C. RIBONUCLEIC ACID

Ten pulvinal sections were homogenized in 10 ml of 0.01 M tris buffer [tris(hydroxymethyl)aminomethane] at pH 7.5 with a VirTis homogenizer for 2 minutes and filtered through Miracloth. A 6-ml portion of the filtrate was made 0.2 N with respect to $HClO_4$ and centrifuged at 2,000 x g for 10 minutes. The pellet was washed (i) at 0 to 4 C once with 0.2 N $HClO_4$ and twice with 0.05 M formic acid in methanol and (ii) at 37 C with ether: ethanol:chloroform (2:2:1 v/v/v) for 30 minutes; then it was hydrolyzed in 0.3 N KOH for 18 hours at 37 C. After cooling, sufficient 2.4 N $HClO_4$ was added to give a final concentration of 0.2 N $HClO_4$. This suspension was centrifuged at 4,000 x g for 10 minutes to yield a clear supernatant. Optical density of the supernatant was measured at 260 and 290 mμ. RNA was calculated by the relationship: mg RNA = (OD at 260 mμ - OD at 290 mμ) x (dilution factor) x (0.048).

D. PROTEIN

After samples of tissue were homogenized and filtered as described above for RNA determinations, 0.7 ml of 50% trichloroacetic acid was added to a 7-ml portion to coagulate the protein. The protein was precipitated by centrifuging at 2,000 x g for 10 minutes and washed (i) at 0 to 4 C first with 5% trichloroacetic acid and then with ethanol and (ii) at 60 C for 5 minutes with ethanol:ether (3:1 v/v). The washed pellet was solubilized in 1 ml of 0.1 N NaOH. The protein content of the NaOH solution was determined by the method of Lowry et al.[9]

E. RIBONUCLEASE

To determine ribonuclease [polyribonucleotide-2-oligonucleotide transferase (cyclizing) EC. 2.7.7.16] the pulvinal portion of 10 explants was homogenized by a VirTis homogenizer at 0 C in 10 ml of 0.05 M potassium acetate buffer, pH 6.5. The homogenate was filtered through Miracloth; then 5 ml of the filtrate were incubated at 37 C with 5 ml of RNA (soluble yeast ribonucleic acid*) solution (1 mg RNA per ml in 0.05 M acetate buffer, pH 6.5). After 2 hours, 20 ml of cold 5% $HClO_4$ in ethanol were added to stop the reaction, and the unhydrolyzed RNA was coagulated by keeping the mixture at -10 C for 1 hour. The suspension of coagulated protein and RNA

* CalBiochem Corp., 3625 Medford St., Los Angeles, California.

was cleared by centrifuging at 20,000 x g for 20 minutes at 0 C. The hydrolyzed RNA in the supernatant was estimated by subtracting the optical density at 290 mμ from the optical density at 260 mμ.

In experiments comparing soluble versus particulate ribonuclease, 10 pulvini were homogenized in 20 ml of buffer (0.36 M sucrose, 1×10^{-3} M EDTA, 0.05 M potassium phosphate, pH 6.5). The homogenate was filtered through Miracloth and 14-ml portions were centrifuged at 20,000 x g for 20 minutes. The sediment was discarded and the supernatant was centrifuged at 105,000 x g for 1.5 hours. The resultant sediment was suspended in a Triton X-100 solution (10 ml of 1×10^{-3} M EDTA, 0.05 M potassium phosphate, pH 6.5, and 0.1% Triton X-100). Five-milliliter portions of this suspension were used in the ribonuclease assay described above. Five-milliliter portions of the 105,000 x g supernatant were assayed directly for ribonuclease activity.

III. RESULTS

In an earlier paper[2] we reported that inhibitors of aging retarded the loss of chlorophyll, RNA, and protein from the pulvinal tissue of bean petiole explants. Although direct evidence was not available, we assumed that the degradative changes occurring in the pulvinus of explants were the result of catabolic enzyme activity. These enzymes either are newly formed or are already synthesized, requiring only release or addition of a prosthetic group for activation. One way to select between these alternatives is to measure the effect of inhibitors of RNA or protein synthesis on the decrease of metabolites in explant pulvinal tissue. If newly synthesized enzymes are necessary for aging, blocking RNA or protein synthesis should prevent the degradative changes associated with aging.

Explants were treated by injecting 1 μliter water or a 1-μliter solution containing either 1 μg of actinomycin D or 0.25 μg of cycloheximide into the pulvinus with a microliter syringe after excision of the explants. Bottles containing explants were vented after 7 hours, and, after 24 hours, the changes in metabolites were measured. Table 1 shows that actinomycin D and cycloheximide did reduce the loss of weight, chlorophyll, RNA, and protein in explant pulvinal tissue.

TABLE 1. INHIBITION OF PULVINAL SENESCENCE BY ACTINOMYCIN D AND CYCLOHEXIMIDE

	% Initial Value after 24 Hours			
	Actinomycin D		Cycloheximide	
Parameter	Control	Inhibitor	Control	Inhibitor
Dry weight	87	93	85	88
Chlorophyll	75	78	62	87
RNA	79	84	67	97
Protein	90	96	76	105

Loss of RNA from pulvinal tissue is an indication of aging in abscission zone explants. Figure 1 shows that the RNA content of the pulvinus decreased slowly for the first 20 hours and then decreased more rapidly. Figure 1 also shows that the soluble ribonuclease activity remained constant for 1 hour, increased during the next 3 hours, remained constant until 16 hours, and then increased again until the experiment was terminated at 24 hours. Alpha-naphthaleneacetic acid (NAA) added to the pulvinus blocked the second increase in ribonuclease, but it did not block the first. The amount of ribonuclease associated with the particulate fraction remained constant during the experiment, suggesting that the increase in soluble ribonuclease was not due to the breakdown of lysosomes, which presumably are in the particulate fraction. The first increase in ribonuclease activity appeared similar to the wounding response observed by Bagi and Farkas.[10] An increase in ethylene production usually follows wounding of bean petiole explants,[7] and it was of interest to see if the initial rise of ribonuclease activity was a response to increased ethylene production. However, ethylene had no effect on the ribonuclease content of pulvinal tissue.

We reported earlier[2] that inhibitors of aging such as indoleacetic acid (IAA), cytokinin SD 8339 [N^6-benzylamino-9,2-(tetrahydropyranyl-9H-purine)], and coumarin retarded the loss of RNA from pulvinal tissue. Table 1 shows that actinomycin D or cycloheximide also prevented the loss of RNA from the pulvinus. If the action of these compounds is to repress the synthesis of ribonuclease at the level of repression, translation, or transcription, then these compounds also should block the increase in pulvinal ribonuclease activity. Data in Table 2 favor this idea because amounts of the various inhibitors sufficient to inhibit abscission also inhibited the formation of ribonuclease.

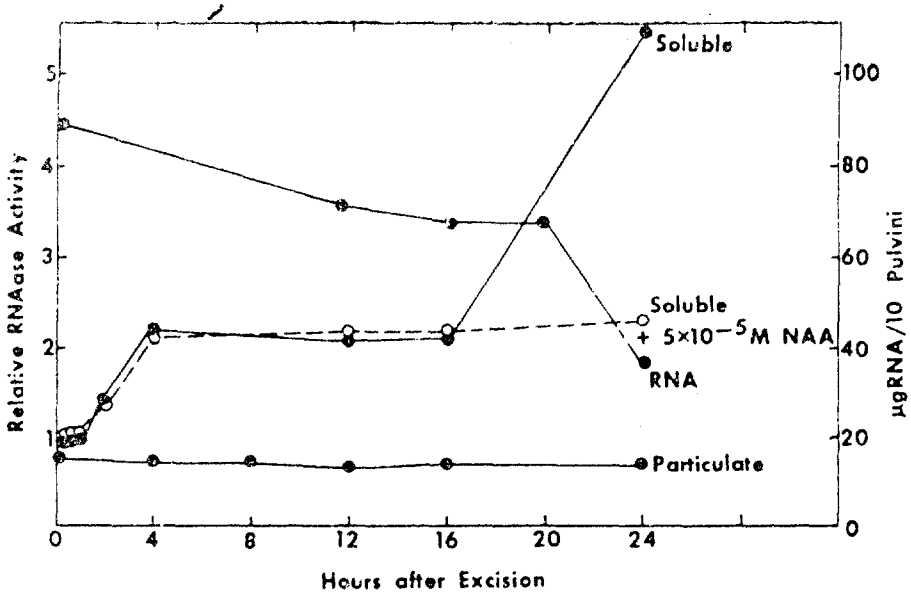

Figure 1. Change in RNA and Ribonuclease Content of Pulvinal Tissue after Excision. Particulate ribonuclease is the activity associated with the material sedimenting between 20,000 x g for 20 minutes and 105,000 x g for 1.5 hours. Soluble ribonuclease is the activity associated with the 105,000 x g supernatant. Initial RNA content of 10 pulvini equaled 72 µg.

TABLE 2. CHANGE IN RIBONUCLEASE LEVELS IN THE PULVINUS OF AGING EXPLANTS[a]

Treatment	Abscission, %	Increase in RNAase after 24 Hours ± SE, %
Control	80	126±3
Coumarin	30	84±6
Cytokinin SD 8339	10	52±1
IAA	0	79±4
Actinomycin D	0	76±3
Cycloheximide	0	17±7

a. Explants were placed, pulvinal end down, in plain agar or agar containing 5×10^{-5} M IAA, 2×10^{-4} M SD 8339, or 2×10^{-4} M coumarin. One μg of actinomycin D or 0.25 μg of cycloheximide in a 1-μliter solution was injected into the separation zone of the explant with a microliter syringe.

IV. DISCUSSION

Earlier work[1,2] has shown that pulvinal tissue loses RNA, chlorophyll, and protein after the excision of bean petiole explants. The loss of these metabolites was prevented by treating pulvinal tissue with IAA, cytokinins, or coumarin,[2] and we concluded that excision of the explant cuts off the supply of aging retardants normally provided by the leaf blade. Although auxin is usually thought to be the compound supplied by the leaf, conclusive chemical evidence is not available. The best evidence shows that the supply of growth-promoting factors is lost from leaves as they age. This aspect of abscission has been discussed in a number of recent reviews.[11-13]

Stimulation of abscission by ethylene depends on an aging process,[7,8] and the action of the gas seems to be through RNA-dependent protein synthesis.[6,14] Although the cell separation process is probably enzymatic, the specific enzymes involved—pectinases, hemicellulases, or cellulases—have not been defined.

Work presented here is centered on understanding the induction of catabolic enzymes in the pulvinus. The loss of metabolites from the pulvinus is mediated by various catabolic enzymes, including chlorophyllases, proteases, and ribonucleases. To some extent, the loss is influenced by

proximity of a subtending metabolic sink, the petiole.[1,2] These catabolic enzymes are either newly synthesized or are preformed and require release or activation before they become effective.

Release of acid hydrolases from cell organelles (lysosomes) has been described for animal tissue undergoing senescence.[15] The existence of similar particles in plant tissue is unresolved, and evidence both in favor of[16-19] and against[20-22] this concept has been advanced.

However, the data in Table 1 favor the concept that the catabolic enzymes appearing in the pulvinus are newly synthesized and are not derived from lysosomes. Using essentially similar techniques, Bagi and Farkas[10] and Kessler et al.[23] concluded that senescence of tobacco and bean leaves also depended on de novo synthesis of degradative enzymes.

Because experimentation with inhibitors is relatively simple, it is a favorite means of determining whether protein synthesis is involved in any specific process. Results with inhibitors are not uniquely interpretable, so that demonstration of increased enzymatic activity that is reduced in the presence of the inhibitor is necessary to show that protein synthesis is involved.

Evidence that levels of ribonuclease increase during aging is shown in Figure 1. After an initial lag of 1 hour, ribonuclease activity increased until 4 hours, after which the level remained constant. A second increase in activity was observed after 16 hours. Loss of RNA from the pulvinus does not occur until after 10 hours (Fig. 1), suggesting that the net loss of RNA is probably associated with enzymes formed during the second increase after 16 hours. Addition of auxin, known to prevent the loss of RNA from the pulvinus,[1,2] also prevented the increase in ribonuclease activity at 16 hours. Additional examples of the inhibition of ribonuclease by the aging retardants cytokinin and coumarin are shown in Table 2. A similar result was shown by Sahai Srivastava and Ware,[24] using detached barley leaves. They found that 6-furfurylaminopurine retarded the loss of chlorophyll, RNA, and DNA and suppressed the activity of deoxyribonuclease and ribonuclease.

Examination of the cell fraction normally thought to contain lysosomes revealed some ribonuclease activity. However, the amount of particulate ribonuclease remained constant during the experiment; if this particulate fraction was the source of the soluble enzyme, then decreases in particulate ribonuclease should have occurred during periods when soluble ribonuclease was formed. A similar result was reported by Balz[17] with ribonuclease and acid protease from aging tobacco leaves. Additional support for the idea that soluble ribonuclease is newly synthesized was obtained when we found that actinomycin D or cycloheximide inhibited the increase in ribonuclease activity (Table 2).

Our ideas concerning events that occur during abscission are summarized in Figure 2. Excision of bean petiole explants cuts off the supply of aging retardants normally supplied by the leaf. Although auxin is the compound usually proposed for this role, others, such as cytokinins and coumarin, should be considered. At some point, the amount of aging retardant in the tissue falls to a point that permits aging to take place. The events occurring during aging are not completely known, but degradative processes, mobilization, and increasing sensitivity to ethylene have been described. Stage 1 (Fig. 2) is defined as the time from excision of explants until the onset of sensitivity to ethylene. Stage 2 starts when explants respond to ethylene. Yamaguchi[5] and Abeles and Rubinstein[7] found that ethylene had no effect on cell separation immediately after excision, but it accelerated the process after a period of aging. Similarly Abeles and Holm[14] found that the gas had no effect on protein synthesis during stage 1 but accelerated the incorporation of leucine-C^{14} into protein during stage 2. Present evidence suggests that ethylene promotes the synthesis of enzymes during stage 2 and that these as yet uncharacterized enzymes are responsible for cell separation. However, other workers[25,26] do not feel that protein synthesis is an integral part of the cell separation process.

Our results suggest that aging retardants normally repress the synthesis of catabolic enzymes, as well as the sensitivity of the separation layer to ethylene. Another example of repression by auxin, that of peroxidase, has been reported by Ockerse et al.[27] Once the concentration of retardants drops below a certain level, catabolic enzymes are synthesized, the tissue becomes sensitive to ethylene, and the gas, in turn, causes the synthesis of cell separation enzymes.

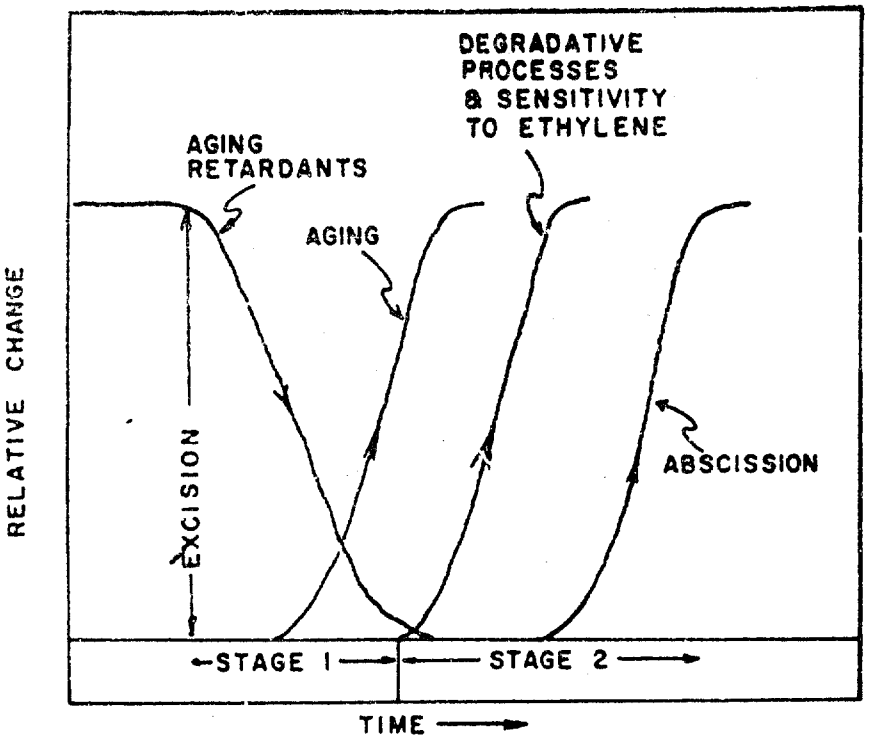

Figure 2. Changes occuring in bean abscission zone explants as a function of time following excision.

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13. ABSTRACT

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14. Key Words

*Abscission
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